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Analytical Performance of a Cell-free DNA Targeted Methylation Test for Early Lung Adenocarcinoma (LUAD) Recurrence Prediction

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INTRODUCTION

- As early detection of non-small cell lung cancer (NSCLC) increases with improved screening for high-risk individuals, methods for assessing recurrence risk may facilitate intervention and improve outcomes
- Cell-free DNA (cfDNA) derived from tumors (ie, circulating tumor DNA [ctDNA]) has emerged as a promising prognostic biomarker across multiple indications[1]
  - In early-stage NSCLC, evidence suggests that ctDNA levels at diagnosis are prognostic for long-term outcomes[2]
- The GRAIL technology platform test uses targeted methylation sequencing of cfDNA and machine learning classifiers (covering >10[5] genomic regions and >1 million CpG sites) to capture the extent and location of tumor-derived methylation in cfDNA from peripheral blood samples and provides a Tumor Methylation Score
- The prognostic test leverages the biological observation that detectable levels of ctDNA in the blood are indicative of proliferative and possibly aggressive cancers, including lung adenocarcinomas (LUAD),[2,3] and an early version of this test demonstrated prognostic capability across cancers[1]
- We developed an optimized Lung Prognosis test for risk of recurrence that requires only a blood draw and no tissue sample. The test utilizes a new version of the methylation-based classifier to identify an individual as having high or low risk of recurrence based on the Tumor Methylation Score
  - ctDNA positive = ctDNA high = Tumor Methylation Score above threshold / high risk of recurrence
  - ctDNA negative = ctDNA low = Tumor Methylation Score below threshold / low risk of recurrence
- The classifier of Lung Prognosis test to compute tumor methylation score was developed using samples from GRAIL's clinical studies
  - The cutoff for optimum prognostic discrimination from this tumor methylation score was selected in an independent cohort of stage I lung cancer cases
- We separately estimate the tumor methylated fraction (TMeF; a methylation-based quantification of the circulating tumor allele fraction and estimate of ctDNA abundance) to characterize the analytical performance of this methylation-based classifier

OBJECTIVE

- Present the analytical validation of a locked tissue-free, plasma-only Lung Prognosis test for risk of recurrence
  - Characterize key assay performance features of the test, including sensitivity, specificity, precision, input titration, interferent effect, and whole blood stability

KEY RESULTS: TISSUE-FREE LUNG PROGNOSIS TEST DEMONSTRATED ROBUST ANALYTICAL PERFORMANCE AT LOW cfDNA INPUT MASS IN EARLY-STAGE LUNG ADENOCARCINOMA

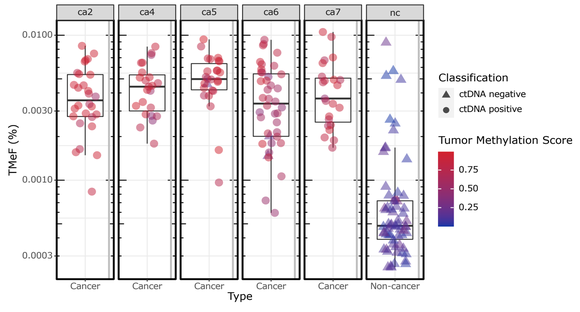
Analytical Sensitivity & Specificity

- Median LoD95 was empirically determined at 0.0044% TMeF (range: 0.0039–0.0052%), or 44 parts per million (PPM), from 5 unique LUAD samples tested
  - The observed value is within ½- to 2-fold of the in-silico determined LoD95 values from a broader set of samples
- Analytical sensitivity evaluated from 140 replicates was 97.9% (95% CI: 93.9%–99.3%) at 6 ng cfDNA input
  - Analytical sensitivity at a typical cfDNA input (20 ng) was 100% with median LoD95 of 0.0073% TMeF (from 56 replicates), which was not significantly different than the sensitivity measured at 6 ng cfDNA
- Analytical specificity LoB of the test was 96.9% (95% CI: 89.6%–99.2%) from 66 replicates at >40 ng non-cancer cfDNA input, with a median TMeF of 0.0005% (range: 0.0003%–0.0089%)
  - The LoB study measures the analytical specificity of the test from healthy individuals; it does not demonstrate the clinical specificity of this Lung Prognosis test because the intended patient population will be clinically diagnosed with cancer
- The estimated median TMeF from non-cancer samples was 10-fold lower than that of LUAD samples near the LoD95 (**Figure 1**)

Positive & Negative Precision

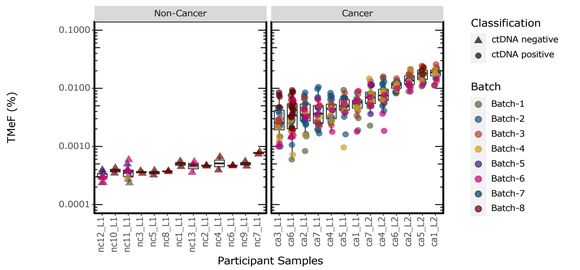
- Positive precision of the test demonstrated 100% repeatability (precision within batch) and 99.1% reproducibility (precision between different batches) for LUAD samples tested at 6 ng cfDNA input
- Negative precision demonstrated 100% repeatability and reproducibility for non-cancer samples tested at 6 ng cfDNA input
- Median TMeF for positive precision was 0.0061%, which is ≈1.4x the LoD95 of the test, and median TMeF for negative precision was 0.0004% (**Figure 2**)

Figure 1. TMeF Distribution of LUAD and Non-Cancer Samples.



TMeF distribution plot by sample type (LUAD vs non-cancer) of all tested LUAD sample replicates near LoD95 and all non-cancer sample replicates from the limit of blank study. Color gradient was used to represent the Tumor Methylation score for both LUAD and non-cancer samples. Classification: ctDNA positive or negative generated by the tissue-free methylation-based classifier. TMeF distribution is represented in a log scale on the y axis.
ca, cancer; ctDNA, circulating tumor DNA; LoD95, limit of detection with ≥95% probability; LUAD, lung adenocarcinoma; nc, non-cancer; TMeF, tumor methylated fraction.

Figure 2. TMeF Was Consistent Within and Between Batches for LUAD and Non-Cancer Samples.



	nc12_L1	nc10_L1	nc11_L1	nc3_L1	nc5_L1	nc8_L1	nc1_L1	nc13_L1	nc2_L1	nc4_L1	nc6_L1	nc9_L1	nc7_L1	ca3_L1	ca6_L1	ca2_L1	ca7_L1	ca4_L1	ca5_L1	ca1_L1	ca7_L2	ca6_L2	ca2_L2	ca5_L2	ca1_L2
Intra-Batch CV(%)	NA	14	NA	7	13	2	15	NA	2	36	1	13	6	35	53	49	46	37	36	35	37	40	NA	19	NA
Inter-Batch CV(%)	17	14	28	7	13	2	15	21	2	36	1	13	6	65	59	49	54	40	35	35	41	43	16	25	23

TMeF distribution of all replicates from positive and negative precision tested at 6 ng cfDNA input. Color was customized for each batch. Intra-batch refers to replicates tested within the same batch, and inter-batch refers to replicates tested across multiple batches. Classification: ctDNA positive or negative generated by the tissue-free methylation-based classifier. TMeF distribution is represented in a log scale on the y axis.
CV, coefficient of variance; L, level; LUAD, lung adenocarcinoma; NA, not applicable; nc, non-cancer; TMeF, tumor methylated fraction.

Input Titration

- Sample quality was 100% evaluable (passed all sample quality specifications, including binary coverage*) with 100% accurate classification across a range of 2 to 75 ng cfDNA input (**Figure 3**)
- At input levels of 0.25 and 0.5 ng, 59 of 62 total replicates were correctly classified, but 57 of these 59 replicates failed the binary coverage quality threshold* (**Figure 3**)

*Binary coverage serves as a control for baseline test performance by quantifying the mean coverage of target regions not expected to be differentially methylated between cancer and non-cancer cfDNA.

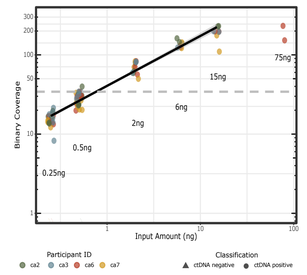
Endogenous Interfering Substances

- Endogenous interferents that were evaluated did not affect quality metrics (evaluability) nor classification accuracy when spiked into contrived cancer samples at 3 to 600x the normal expected range, per Clinical and Laboratory Standards Institute (CLSI) guidelines[4] (**Figure 4**)

Whole Blood Stability

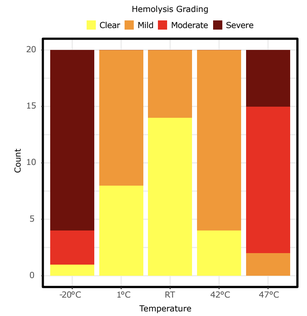
- Whole blood from non-cancer donors was stable at temperatures ranging from 1ºC to 42ºC, with no severe hemolysis, ≥95% samples evaluable, and ≥94% classification accuracy
- At -20ºC, 80% of samples had severe hemolysis, and at 47ºC, 25% of samples had severe hemolysis (**Figure 5**)
  - Despite severe hemolysis, all samples (N = 20) from 47ºC and 9 samples from -20ºC were processed through the test workflow to assess the effect on sample evaluability and classification; this is not typical of the production workflow. 90% of samples at 47ºC and 100% of samples at -20ºC were evaluable, and both sets had 100% classification accuracy
- The test conditions (-20ºC, 1ºC, 42ºC, 47ºC) had 94.7%–100% pairwise concordance with the reference condition (room temperature: 19ºC–25ºC) (**Figure 6**)
- Whole blood from non-cancer donors was stable at room temperature up to 8 days, with ≥95% samples evaluable and ≥95% classification accuracy. The test condition (Day 8) showed ≥95% pairwise concordance with the reference condition (Day 1) (**Figure 6**)

Figure 3. Samples Were Correctly Classified Across a Range of cfDNA Input Amounts.



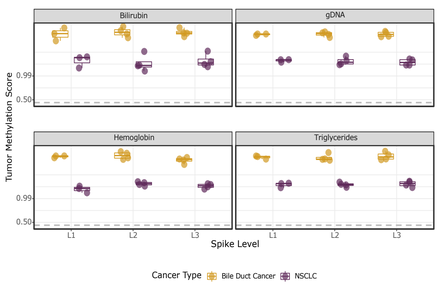
Regression between cfDNA input amount and binary coverage of all tested replicates from the input titration analysis. Log-log linear regression was applied to the input range of 0.25–15 ng, shown as black solid line with the gray area of 95% confidence interval. The binary coverage threshold is shown as a gray dashed line. Classification: ctDNA positive or negative generated by the tissue-free methylation-based classifier. Binary coverage is represented in a log scale on the y axis.
ca, cancer; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA.

Figure 5. Most Samples Within the Range of 1ºC to 42ºC Had Mild or No Hemolysis.



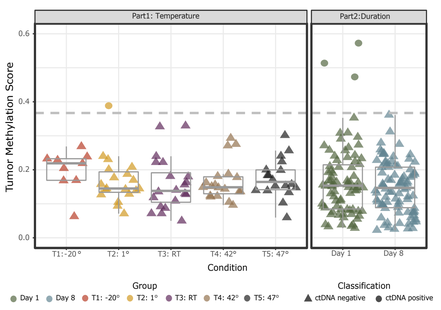
Hemolysis grading of samples with different shipping temperatures.
RT, room temperature.

Figure 4. Endogenous Interferents Did Not Cause Misclassification of LUAD Samples.



Tumor methylation score of 2 contrived cancer samples (bile duct cancer and NSCLC; analytical performance is not affected by cancer type) spiked with 4 different interferents at 2 different spike levels. L1, unspiked baseline; L2, low level; L3, high level. Tumor methylation score threshold to identify LUAD with higher risk of recurrence is shown as a gray dashed line. Tumor methylation score is represented in a logit scale on the y axis.
gDNA, genomic DNA; L, level; LUAD, lung adenocarcinoma; NSCLC, non-small cell lung cancer.

Figure 6. Temperature Conditions and Storage Times Had No Impact on Classification of Non-Cancer Samples.



Tumor methylation scores of non-cancer samples shipped or stored under different conditions. Tumor methylation score threshold is shown as a gray dashed line. Classification: ctDNA positive or negative generated by the tissue-free methylation-based classifier.
ctDNA, circulating tumor DNA; RT, room temperature; T, temperature.

CONCLUSIONS

- Results of these key performance features demonstrate robust analytical performance of a blood-based, tissue-free, cfDNA-based targeted methylation assay for predicting risk of LUAD recurrence (**Table 1**)
- Future studies will evaluate the clinical utility of recurrence risk assessment by ctDNA detection above a pre-specified threshold in early LUAD

Table 1. Overview of Analytical Performance of Lung Prognosis Test for Risk of Recurrence.

	Analytical Performance	Median TMeF (Range)
Analytical Sensitivity (Limit of Detection)[a]	97.9% (95% CI 93.9–99.3%)	0.0044% (0.0039–0.0052%) or 44 PPM
Analytical Specificity (Limit of Blank)[b]	96.9% (95% CI 89.6–99.2%)	0.0005% (0.0003–0.0089%)
Positive Precision[c]	Repeatability: 100% Reproducibility: 99.1%	0.0061% (0.0006–0.0259%)
Negative Precision[d]	Repeatability: 100% Reproducibility: 100%	0.0004% (0.0002–0.0008%)
Input Titration[e]	100% QC pass with accurate classification within 2–75 ng	NA
Interfering Substance[f]	100% QC pass with accurate classification at 3–600x normal levels	NA
Whole Blood Stability[g]	Temperature: 1–42 ºC Duration: Up to 8 days	NA

[a]140 replicates from 5 LUAD participants at empirical LoD95 level.
[b]66 replicates from 62 non-cancer participants.
[c]325 replicates from 7 LUAD participants at 0.7–4x assay LoD95 level.
[d]43 replicates from 9 individual and 3 pooled non-cancer participants.
[e]Note, there is no minimum cfDNA input requirement for the assay.
[f]Interferent highest levels relative to normal range.
[g]Shipping temperature and storage duration with no severe hemolysis, ≥95% quality evaluability and ≥94% classification accuracy.
cfDNA, cell-free DNA; CI, confidence interval; LoD95, limit of detection with ≥95% probability; LUAD, lung adenocarcinoma; NA, not applicable; PMM, parts per million; QC, quality control TMeF, tumor methylated fraction.

METHODS

Study Samples

- Analytical performance evaluation of the GRAIL blood-based, tissue-free Lung Prognosis test for risk of recurrence, which was validated with LUAD samples that were prospectively collected and retrospectively tested, used cfDNA samples from 100 healthy, non-cancer donors and 7 individuals with clinically diagnosed, treatment-naive early-stage (II-III) LUAD. An additional 66 male and 53 female healthy, non-cancer donor samples were used to prepare background cfDNA for dilutions
- All samples were collected in accordance with AstraZeneca human biological samples and informed consent policies

Analytical Sensitivity

- Analytical sensitivity [LoD] was defined as the limit of detection with ≥95% probability (LoD95) of signal detection at the lowest TMeF
- LUADs were selected from biobank for the study based on 2 criteria: sufficient material availability and in-silico LoD95 estimates from prior sequencing results
  - In-silico results from 19 samples demonstrated the LoD95 estimates to range from 0.0021 to 0.0086% TMeF (Stage I, II and III)
- LoD95 was measured individually from separate dilution series of 5 unique LUAD cfDNA samples spiked into a background of pooled, gender-matched, non-cancer cfDNA to create 5 to 6 dilution levels at 6 ng total cfDNA input
- A similar dilution series of 3 LUAD cfDNA samples was used to evaluate the analytical sensitivity at 20 ng cfDNA input, but with fewer replicates at each level than tested at 6 ng cDNA input due to limited sample availability
- TMeF of the individual samples or each of the dilution pool used for LoD95 determination was not orthogonally validated due to unavailability of suitable technology that can measure a similar metric

Analytical Specificity

- Analytical specificity [LoB] was assessed as the percentage of samples with ctDNA not detected among 66 valid replicates tested from 62 unique non-cancer samples

Precision

- Positive and negative precision was characterized by concordance with expected ctDNA positive/negative classification in replicates from 7 unique LUAD and 13 unique non-cancer samples at 6 ng cfDNA input

Input Titration

- Classification accuracy was evaluated across a range of cfDNA inputs (0.25–75 ng) from 4 unique LUAD samples, prepared by serial dilution
- Classification accuracy was measured by the percentage of replicates with Tumor Methylation Score above its threshold

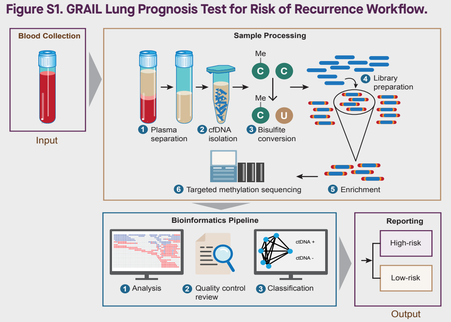
Endogenous Interferents

- Classification accuracy was evaluated across 4 common endogenous interferents spiked at 2 different concentrations with contrived cancer samples (ie, bile duct cancer or NSCLC cfDNA diluted into healthy donor plasma pool)
  - Bilirubin: 10, 20 mg/dL (normal: 0-2 mg/dL); hemoglobin: 500, 2000 mg/dL (normal: <3 mg/dL); triglycerides: 200, 500 mg/dL (normal: <150 mg/dL); genomic DNA (gDNA) extracted from commercially sourced white blood cells: 125%, 500% of total extracted cfDNA from unspiked baseline

Whole Blood Stability

- Whole blood stability in Streck tubes was characterized using summer and winter shipping conditions and extended room temperature stability for up to 8 days. The study utilized 25 unique non-cancer samples

Figure S1. GRAIL Lung Prognosis Test for Risk of Recurrence Workflow.



ctDNA, circulating tumor DNA; Me, methyl group.
Figure adapted from Alexander GE, et al. *PLoS One*. 2023;18(4):e0283001.

References

1. Chen X et al. *Clin Cancer Res*. 2021;27(15):4221-4229.
2. Chabon JJ et al. *Nature*. 2020;580:245-51.
3. Abbosh C et al. *Nature*. 2023;616(7957):553-562.
4. EP37 Supplemental Tables for Interference Testing in Clinical Chemistry. Clinical and Laboratory Standards Institute. 2018.

Disclosures

GRAIL-affiliated authors are employees of GRAIL, LLC, with equity in Illumina, Inc. AstraZeneca-affiliated authors are employees of AstraZeneca and own shares in AstraZeneca.

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